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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Yong Liang CHU, et al. ART UNIT: 1636
SERIAL NO.: 10/035,223 EXAMINER: Ramin Akhavan
FILING DATE: January 4, 2002
FOR: COMPOUNDS FOR DELIVERING SUBSTANCES INTO CELLS

DECLARATION UNDER RULE 1.132

I, Yong Liang CHU, do hereby declare as follows:

I am the first named inventor of the above-identified patent application. Since the filing of the above-identified patent, we have performed a number of experiments using the compounds and methods claimed in the above-identified patent application. Representative experiments are provided in the following Examples.

Example 1: Synthesis of 1-tetradecyl myristoylamide

At room temperature, a solution of 1-tetradecylamine (10.0g, 44.5 mmole) and diisopropylamine (10.8 ml, 62.3 mmole) in 40 ml of anhydrous dichloromethane 40 ml was added slowly into a solution of myristoyl chloride (11.3 g, 44.5 mmole) in 160 ml of dichloromethane with mechanic stirring. A white precipitate was formed. After 48 hours, the reaction mixture was filtered. The white solid was washed twice with methanol. The white solid was dried over vacuum and the filtrate was discarded. A total of 17.1 g of product was obtained. The purity was over 90% based on TLC analysis.

Example 2: Synthesis of di-tetradecylamine

Lithium aluminum hydride powder (2.2 g, 57.8 mmole) was added carefully into a suspension of 1-tetradecyl myristoylamide in 190 ml of THF. With magnetic stirring, the reaction mixture was heated to 70°C. After 8 hours, the reaction mixture was cooled to room temperature, and quenched with 6N NaOH aqueous solution. The mixture was stirred at room temperature for 1.5 hours. After separation, the aqueous portion was extracted with ethyl ether twice with 350 ml each time followed by 400 ml of dichloromethane. The combined organic solutions were washed with saturated sodium chloride, and concentrated to afford 13.2 g of di-tetradecylamine white solid. The purity of the final product was analyzed with 20% methanol in dichloromethane.

Example 3: Synthesis of Ditetradecyl-[2-hydroxyl-3-(N-phthalamido)propyl]amine

Under nitrogen, a mixture of di-tetradecylamine (8g, 19.6 mmole), N(2,3-epoxyl)propyl phthamide (5.17g, 25.5 mmole) and diisopropylethylamine (5.2 ml, 29.4 mmole) in 30 ml dimethylformamide were heated to 75°C for 18 hours with magnetic stirring. After being concentrated, the resulting product was purified with silica gel flash chromatography, eluted with 1% to 5% MeOH in dichloromethane. White solid of Ditetradecyl-[2-hydroxyl-3-(N-phthalamido)propyl]amine was obtained at 70% yield.

Example 4: Synthesis of Ditetradecyl-(2-hydroxyl-3-propylamino)amine

Hydrazine (0.36 g, 11.6 mmole) was added in to a mixture of Ditetradecyl[2-hydroxyl-3-(N-phthalamido)propyl]amine in 30 ml of methanol. The mixture was heated to reflux for 4 hours. After all the starting material was consumed. The mixture was filtered and concentrated to obtain white solid.

Example 5: Synthesis of Ditetradecyl-(2-hydroxyl-3-propylamino)aminopolylysine

N-hydroxysuccinimide (150 mg, 1.3 mmole) and diisocarbodiimide (164 mg, 1.3 mmole) were added into a solution of K(K(BOC)2)2 (1.0 mmole, purchased from Advanced ChemTech) in 3ml DMF and 3 ml dichloromethane. After 1.5 hours stirring at room temperature, a solution of Ditetradecyl-(2-hydroxyl-3propylamino)amine (483 mg, 1 mmole) in 2 ml DMF and 2 ml of dichloromethane was added. The mixture was stirred at room temperature for additional 3 hours, then was concentrated and purified with flash chromatography. The purified product was treated with 25% TFA in DCM for 1 hour, before removing the solvents. The final product was purified with C-18 reverse phase chromatography. A wax-like product was obtained at 65% yield.

Example 6: Synthesis of Octadecyl-[2-hydroxyl-3-(N-phthalamido)propyl]amine

Under nitrogen, a mixture of di-octadecylamine (10g, 19.2 mmole), N-(2,3-epoxyl)propyl phthamide (4.5 g, 22.1 mmole) and diisopropylethylamine (6.2 g, 48 mmole) in 40 ml dimethylformamide were heated to 80°C for 18 hours with magnetic stirring. After being concentrated, the resulting product was purified with silica gel flash chromatography, eluted with 1% to 5% MeOH in dichloromethane. White solid of Dioctadecyl-[2-hydroxyl-3-(N-phthalamido)propyl]amine was obtained at 70% yield.

Example 7: Synthesis of Octadecyl-(2-hydroxyl-3-propylamino)amine

Hydrazine (1.07 ml, 34.2 mmole) was added in to a mixture of Dioctadecyl-[2-hydroxyl-3-(N-phthalamido)propyl]amine (8.3 g, 11.4 mmole) in 100 ml of methanol. The mixture was heated to reflux for 6 hours. After all the starting material was

consumed, the mixture was filtered and concentrated to obtain white solid.

Example 8: Synthesis of Dioctadecyl-(2-hydroxyl-3-propylamino)aminopolylysine

N-hydroxysuccimide (150 mg, 1.3 mmole) and diisocarbodiimide (164 mg, 1.3 mmole) were added into a solution of K(K(BOC)2)2 (1.0 mmole, purchased from Advanced ChemTech) in 3ml DMF and 3 ml dichloromethane. After 1.5 hours stirring at room temperature, a solution of Dioctadecyl-(2-hydroxyl-3-propylamino)amine (595 mg, 1 mmole) in 2 ml DMF and 2 ml of dichloromethane was added. The mixture was stirred at room temperature for additional 3 hours, and was then concentrated and purified with flash chromatography. The purified product was treated with 25% TFA in DCM for 1 hour, and the solvent was removed. The final product was purified with C-18 reverse phase chromatography. A wax-like product was obtained at 71% yield. Mass analysis show MW+1 = 979.93.

Example 9: Formulation of the lipid reagents

The lipid Ditetradecyl-(2-hydroxyl-3-propylamino)aminopolylysine or Dioctadecyl-(2-hydroxyl-3-propylamino)aminopolylysine was formulated with neutral compound such as DOPE, cholesterol via commonly used formulation methods, such as extrusion, vortexing, freeze-thaw, ultrasonic agitation, and microfluidization formulation. The molar ratio between cationic lipid and neutral lipids is 0.1 to 10.

Example 10: Lipid formulation as transfection reagent for DNA delivery into 293 and Hela cells in 24-well format

Plate 293 or Hela cells in 24-well plates at a density of 5 x 10^4 cells/well in 0.5 ml of complete media (DMEM medium + 10% FBS, GIBCO Invitrogen) without antibiotics. Put the

plates in a 37°C, 5% CO_2 humidified incubator. After 24 – 48 hours, the cells reach 60 – 80% confluence, and begin preparations for cell transfection:

a. Dilute 0.8 ug of DNA in 20 ul of deionized H_2O. Mix and spin down the solution for a few seconds to remove drops from the top of the tube.

b. In a separated vial, dilute 1.5, 3, 4.5 6 and 7.5 ul of transfection reagent with deionized H_2O to total 20ul.

c. Add the transfection reagent to the DNA solutions. Mix by pipetting up and down 6 times.

d. Let the solutions incubate for 15 minutes (20 – 25°C).

e. Add 60 ul of DMEM to the DNA-lipid complex to final volume of 100 ul.

f. Add immediately the complex drop-wise to the appropriate well. Gently swirl the plate to ensure uniform distribution of the transfection complexes.

g. Put the cells back into the incubator.

h. Incubate the cells with the tranfection mixtures for 48 hours.

i. The ß-gal protein expression of the transfected 293 and Hela cells were measured using ONPG assay kit (Invitrogen) at 420 nm based on the manufacture's protocol.

The transfection of 293 or Hela cells (24-well plate) at various amount of transfection reagent and pCMV-SPORT-ßgal plasmid DNA. The lipid formulation of the present invention (Dioctadecyl-(2-hydroxyl-3-propylamino)-aminopolysine and DOPE at 1:1 mole ratio designated as "GXP-1") are shown in Figure 1A and Figure 1B. Image of stained 293 cells after transfection is shown in Figure 1C.

Example 11: Lipid formulation as transfection reagent for DNA delivery into COS-7 cells in 96-well format

Plate COS-7 cells in 96-well plates at a density of 2×10^4 cells/well in 0.2 ml of complete media (DMEM medium + 10% FBS, GIBCO Invitrogen) without antibiotics. Put the plates in a 37°C, 5% CO_2 humidified incubator. After 24 hours, the cells reach 60 – 80% confluence, and begin preparations for cell transfection:

a. Dilute 0.2 ug of DNA in 4 ul of deionized H_2O. Mix and spin down the solution for a few seconds to remove drops from the top of the tube.

b. In a separated vial, dilute 0.5, 0.75, 1.0 and 1.5 ul of tansfection reagent with deionized H_2O to total 4 ul.

c. Add the transfection reagents to the DNA solution. Mix by pipetting up and down 6 times.

d. Let the solutions incubate for 15 minutes (20 – 25C).

e. Add 12 ul of DMEM with no serum and no antibiotics to the DNA-lipid complex to final volume of 20 ul.

f. Add immediately the complexes drop-wise to the appropriate well. Gently swirl the plate to ensure uniform distribution of the transfection complexes.

g. Put the cells back into the incubator.

h. Incubate the cells with the tranfection mixtures for 48 hours.

i. The ß-gal protein expressions of the transfected COS-7 cells were measured using ONPG assay kit at 420 nm based on the manufacture's protocol(Invitrogen).

The comparison of the DNA transfection efficiency among GXP-1, two other commercial transfection reagents "Reagent 1", "Reagent 2" and control on COS-7 cells at 96-well plate format are shown in Figure 2.

Example 12: Lipid formulation as transfection reagent for siRNA delivery

For adherent cells: plate the cells in 24-well plates at a density of 5 - 8 x10^4 per well in 0.5 ml of complete media without antibiotics, place plates in a 37°C, 5% CO_2 humidified incubator, and start preparing for transfection after 24-48 hours, when the cells reach 60-80% confluence.

For suspension cells: split the cells the day before transfection. On the day of transfection, harvest cells by centrifugation and remove the medium. Seed 5.5 - 8.5 x10^5 cells per well in 0.5 ml of complete media without antibiotics in 24-well plate, and begin preparation for transfection:

a. Dilute 0.8 ug of DNA in 20 ul of deionized water containing no serum, proteins or antibiotics. Mix and spin the solution for a few seconds to remove drops from the top of the tube.

b. In a separated vial, dilute 3 ul of transfection reagent with deionized water to a final volume of 20 ul.

c. Add the transfection reagent to the DNA solution. Mix by pipetting up and down 6 times.

d. Add siRNA (60 pmole) to the diluted complex mixture.

e. Incubate the solution for 10 minutes at 20 - 25°C.

f. Add 60 ul of DMEM with no serum and no antibiotics to the DNA-lipid complex

to a final volume of 100 ul.

g. Immediately add the complexes drop-wise to the appropriate well. Gently swirl the plate to ensure uniform distribution of the transfection complexes.

h. Put the cells back into the incubator.

i. Incubate the cells with the transfection mixtures for 48 hours, before assay.

The result of an exemplary transfection experiment using siRNA is shown in Table 1.

Table 1: Cotransfection of pCMV-SPORT-βgal plasmid DNA with siRNA using lipid formulation of the present invention (Dioctadecyt-(2-hydroxyl-3-propylamirlo)aminopolylysine and DOPE at 1:1 mole ratio)

DNA (ug)	Reagent (ul)	siRNA (pmols)	Cell transfected
0.8	3	0	93%
0.8	3	60	15%

As shown in Examples 1-11, cationic lipid compounds dioctadecyl-(2-hydroxyl-3-propylamino)aminopolylysine (Example 5) or dioctadecyl-(2-hydroxyl-3-propylamino)aminopolylysine (Example 8), both of which are covered by formula (I) of the above-identified application, were formulated with neutral compound such as DOPE or cholesterol via commonly used formulation methods (Example 9). Effective *in vitro* DNA and siRNA delivery was achieved with the lipid formulation (Examples 10 and 11). It should be noted that the formulation of the lipid reagents, the formation of the DNA-lipid or DNA-siRNA-lipid complex, as well as the transfection process, were all performed under commonly used conditions that are well known to an skilled artisan.

For example, the lipid reagents were formulated with DOPE or cholesterol using extrusion, vortexing, freeze-thaw, or ultrasonic agitation; the molar ratio between cationic lipid and neutral lipids was 0.1 to 10. Those are standard formulation conditions used in the art. Similarly, the transfection was performed using cells at 60-80% confluency (monolayer) or a suspension density of 5.5 - 8.5 x 10^5 cells / 0.5 ml media; the lipid-DNA complexes were formed by incubating the transfection reagent to the DNA solution at room temperature for 15 minutes; the complexes were diluted with DMEM with no serum and no antibiotics, and then added dropwise to the cell. All these procedures and conditions are within the standard range that can be found in most transfection protocols. For example, see Ausubel et al. Short Protocols in Molecular Biology, 2nd Edition, 1992, Section 9.4: Liposome-Mediated Transfection and Section 9.9: Optimization of Transfection; and Promega Inc.'s Transfection Guide, 1998, Chapters 1-3.

The working examples therefore demonstrate that lipids embraced by Claim 1 of the instant application are capable of introducing substances, such as DNA and siRNA, into cells under standard experimental conditions. Since cationic lipids having the general structure of formula (I) in the above-identified application share the basic features of being positively charged under neutral pH and being able to form liposome structures in an aqueous environment under agitation, and since Examples 1-11 have convincingly demonstrated that two of those lipids are capable of introducing foreign molecules into cells, it is my opinion that other lipids embraced by formula (I) would similarly be used to introduce substances into cells using known transfection protocols as of the filing date of the instant application.

I declare that all statement made herein based on my own knowledge are true, and that all statements made herein based on information and belief are believed to be true. I further declare

that these statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United State Code, and that willful false statements may jeopardize the validity of the above-referenced patent application and any patent that issues therefrom.

Date: 7-2-2004

Yong Liang CHU

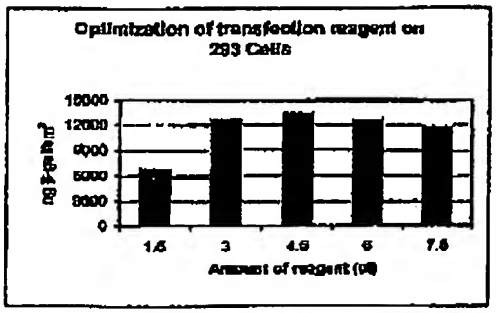

Fig 1A. GXP-1 Transfection reagent, pCMV-SPORT-βgal DNA 0.8 ug, 5 x 10^4 293cells, 24-well plate, 10% FBS.

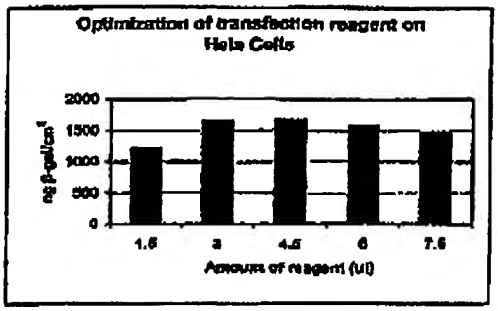

Fig 1B. GXP-1 Transfection reagent, pCMV-SPORT-βgal DNA 0.8 ug, 5x10^4 Hela cells, 24-well plate, 10% FBS.

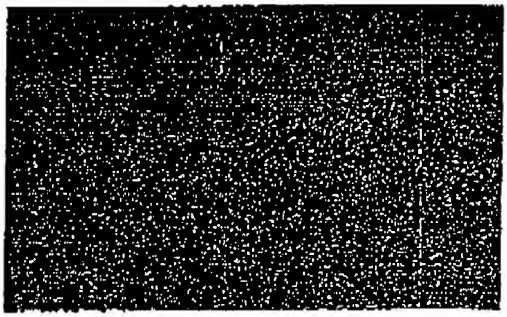

Fig. 1C Image of stained 293 cells after transfection with 4.5 ul of GXP-1 and 0.8 ug of pCMV-SPORT-βgal DNA. The blue stained cells are DNA transfected cells containing βgal protein. Majority of 293 cells have been transfected by GXP-1/βgal DNA complex with strong βgal protein expression.

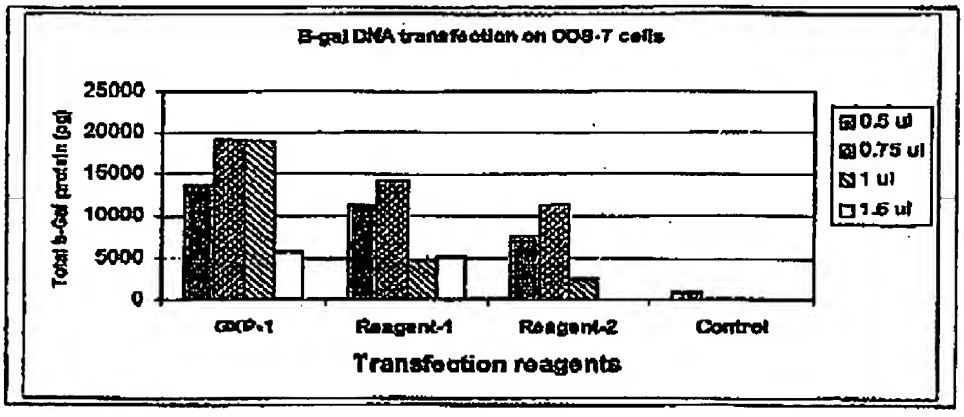

Fig 2. GXP-1 Transfection reagent, 0.2 ug pCMV-SPORT-βgal DNA, 2 x 10^4 COS-7 Cells, 96-well plate, with 10% FBS.